REMARKS

Objection to the Amendments under 35 U.S.C. §132

Rejection of Claims 1-11 and 35-43 under 35 U.S.C. §112, first paragraph

The Examiner states that the "amendment filed 12/27/99 remains objected to and the amendment filed 8/18/00 is objected to under 35 U.S.C. 132 because it introduces new matter into the disclosure" (Office Action, page 2). The Examiner states that the "added material which is not supported by the original disclosure is as follows: 'wherein the site of the naturally occurring deletion is not site III' and 'wherein the naturally occurring deletion site is selected from the group consisting of" site I, site II, site IV, site V and site VI'" (Office Action, pages 2-3).

Claims 1-11 are rejected under 35 U.S.C. §112, first paragraph "as containing subject matter which was not described in the specification in such a way as to reasonably convey to one skilled in the relevant art that the inventor(s), at the time the application was filed, had possession of the claimed invention" (Office Action, page 3). The Examiner states that "Claims 1-11 and 35-43 stand rejected as containing new matter, as the new subgenus of deletion sites either a) specifically excluding site III; or b) reciting all sites except site III, do not find basis in the specification as filed" (Office Action, page 3).

The Examiner states that the specification "sets forth that the invention is drawn to MVA viruses having a heterologous antigen inserted into a (any) naturally occurring deletion site of that virus, of which there are six" (Office Action, page 3). The Examiner notes that "Applicant is now claiming a new subgenus of MVA virus wherein the site is not site III" and that "[t]his new subgenus is not identified as a preferred subgenus in the specification, nor are any particular problems with site III set forth such that one of ordinary skill in the art would reasonably assume that the new subgenus of viruses was intended as the invention" (Office Action, page 3).

Applicants respectfully disagree. In the specification as filed, Applicants teach that:

> According to the present invention a foreign DNA sequence was recombined into the viral DNA precisely at the site of a naturally occurring deletion in the MVA genome (specification, page 6, lines 21-24)

and that:

> Six major deletions of genomic DNA (deletion I, II, III, IV, V, and VI) totaling 31,000 base pairs have been identified (specification, page 5, lines 14-16).

Clearly, one of ordinary skill in the art would assume that recombinant MVA containing and capable of expressing at least one foreign gene inserted in any one of the six deletion sites of MVA was intended as the invention. Applicants claim a subgenus which is clearly disclosed in the specification as filed, not a "new" subgenus. Furthermore, it is not a requirement that a claim to a subgenus be "identified as a preferred subgenus in the specification", or that such a claim be supported in the specification by identifying "any particular problems" with the elements that are not members of the subgenus. As the court points out in *In re Wertheim*,

> Inventions are constantly made which turn out not to be patentable, and applicants frequently discover during the course of prosecution that only a part of what they invented and originally claimed is patentable. . . . To rule otherwise would let form triumph over substance, substantially eliminating the right of an applicant to retreat to an otherwise patentable species merely because he erroneously thought he was first with the genus when he filed. (Emphasis in original). In re Wertheim at 97.

Subsequent to the filing of the instant application, it was discovered that Sutter *et al.* 1994 (Reference AR, PTO form 1449) describe plasmids in which the hemagglutinin (HA) and nucleoprotein (NP) genes are inserted into the deletion site III of the MVA virus (Sutter *et al.*, page 1032, column 2; Figure 1). Applicants amended their claims to be free of such art, which the courts have clearly indicated is allowable.

The Examiner acknowledges Applicants citation of *In re Wertheim*, but states that "the facts in the instant application can be distinguished from Wertheim, and as such, Applicant's conclusions are unpersuasive" (Office Action, page 3). The Examiner states that "In re Wertheim is concerned with concentrations of compound in a composition, and whether a particular value within a described range is also described" and that "[t]his is different from the instant claims, as the claims are drawn to exclude a specific embodiment from a genus" (Office Action, page 3).

It is a rare exception when the facts of a case cannot be distinguished from the facts of the instant application. Applicants cite *In re Wertheim* as support for the right of an applicant to retreat to an otherwise patentable species. Applicants also cited *In re Johnson and Farnham*,

194 U.S.P.Q. 187 (C.C.P.A. 1977), wherein the court found that Appellants' *exclusion of two species from an original genus claim to avoid having the claims read on a lost interference count was supported by the specification*. The court stated that:

> . . . .Appellants . . . are narrowing their claims, and the full scope of the limited genus now claimed is supported in appellants' earlier application, generically and by specific examples.
>
> The notion that one who fully discloses, and teaches those skilled in the art how to make and use, a genus and numerous species therewithin, has somehow failed to disclose, and teach those skilled in the art how to make and use, ***that genus minus two of those species***, and has thus failed to satisfy the requirements of § 112, first paragraph, appears to result from a hypertechnical application of legalistic prose relating to that provision of the statute. (*In re Johnson and Farnhan*, 194 U.S.P.Q. 187, 196 (C.C.P.A. 1977), emphasis added).

However, the Examiner has not addressed this court decision.

Citing *Ex Parte Klager*, the Examiner states that "[i]t is well settled that Applicant may not claim a specific thing not originally described merely because it comes within the scope of the genus disclosed" (Office Action, page 4).

In *Ex parte Klager*, appellant did not have literal support for the term in a claim (*i.e.*, 1-chloro-1-nitroethylene). In contrast, and as pointed out above, Applicants have specifically described the claimed invention in the original specification. In the specification as filed, Applicants teach that "[a]ccording to the present invention a foreign DNA sequence was recombined into the viral DNA precisely at the site of a naturally occurring deletion in the MVA genome" (specification, page 6, lines 21-24) and that "[s]ix major deletions of genomic DNA (deletion I, II, III, IV, V, and VI) totaling 31,000 base pairs have been identified (specification, page 5, lines 14-16).

Citing *In re Welstead*, *In re Ruschig*, *In re Smith*, and *Ex Parte Westphal*, the Examiner states that "[a]rbitrarily designating a group of materials subgeneric to the group previously claimed which was not delineated or supported as such does not have basis" (Office Action, page 4). The Examiner states that the "group originally claimed was the group of recombinant viruses having an insertion in *a (any)* naturally occurring deletion site of the virus" and that a "*specifically disclosed* subgenus was the subgenus of MVA viruses having an insertion into the naturally occurring deletion site, site II" (Office Action, page 4).

As Applicants teach in the specification as filed, and as known in the art, a naturally occurring deletion site in the MVA genome (*i.e.*, the group or genus) is one of six deletion sites, designated I, II, III, IV, V and VI. Based on what those of skill in the art know about the MVA virus, Applicants respectfully disagree that designating each deletion site as a subgenus of the group can hardly be considered arbitrary. As the Examiner points out, Applicants *specifically disclose* the group (genus):

> According to the present invention a foreign DNA sequence was recombined into the viral DNA precisely at the site of a naturally occurring deletion in the MVA genome (specification, page 6, lines 21-24)

In addition, Applicants *specifically disclose* materials subgeneric to the group:

> Six major deletions of genomic DNA (deletion I, II, III, IV, V, and VI) totaling 31,000 base pairs have been identified (specification, page 5, lines 14-16).

Furthermore, Applicants *specifically exemplify* a subgenus of MVA viruses having an insertion into the naturally occurring deletion site II.

Clearly, the specification as filed supports specifically excluding site III and reciting all sites except site III. A person skilled in the art would recognize from the specification, as originally filed, that Appellants had possession of the presently claimed recombinant MVA viruses at the time the subject application was filed.

Applicants have not introduced new matter into the referenced application. In addition, Applicants have fully satisfied the requirements of 35 U.S.C §112.

Rejection of Claims 1-5, 11, 35-38 and 44-47 under 35 U.S.C. §103(a)

Claims 1-5, 11, 35-38 and 44-47 are rejected under 35 U.S.C. §103(a) "as being unpatentable over Small Jr. et al. (US Patent 5,676,950)" (Office Action, page 5). The Examiner states that Small *et al.* disclose "recombinant MVA viruses wherein antigenic determinants from influenza or from HIV are ***inserted into a naturally occurring deletion of the MVA virus***" and that "MVA is a preferred virus due to its extreme attenuation, yet unimpaired gene expression" (Office Action, page 5). It is the Examiner's opinion that the disclosure of Small *et al.* that heterologous gene expression is unimpaired is a clear indication that MVA viruses having

insertions into one of these six deletion sites are able to express that heterologous gene" and that "that is the thrust of the entire invention . . .as they are concerned with the expression of influenza antigens from recombinant MVA viruses" (Office Action, page 5, emphasis added). The Examiner states that "Applicant attempts to cast doubt as to where the heterologous sequences of Small Jr. et al. were inserted, but provides no other evidence that those sequences were not inserted into any of the naturally occurring deletion sites" (Office Action, page 5).

Applicants respectfully disagree. Applicants have provided evidence that Small *et al.* do not disclose "recombinant MVA viruses wherein antigenic determinants from influenza or from HIV are inserted into a naturally occurring deletion of the MVA virus". Indeed, the Examiner clearly states that:

> ***Small Jr. et al. do not specifically identify which insertion site is used in their recombinant viruses*** (Office Action, page 6, emphasis added).

More specifically, and as Applicants point out in the previously filed Amendment, Small *et al.* do not specifically disclose how the MVA HA-NP was constructed. Thus, Small *et al.* do not disclose where the HA-NP was inserted in the MVA genome. Furthermore, those of skill in the art know that a foreign sequence can be inserted into numerous non-essential regions of the MVA genome wherein heterologous gene expression is unimpaired. At the time of Applicants' invention it was known that heterologous genes could be inserted into non-essential regions of the vaccinia virus. A vaccinia virus genome comprises numerous non-essential gene which are used as insertion sites of foreign genes. It is likely that Small *et al.* inserted the heterologous gene into the C7L- locus, the tk-locus or one of the other 35 sites in the vaccinia virus genome into which foreign genes can be inserted, and which are also still present in the MVA genome (see Table 1 of Smith, *Molecular Virology; A Practical Approach*, Eds. A.J. Davison and R.M. Elliot, IRL Press at Oxford University Press, a copy of which is being filed herewith as Exhibit A). Insertion of a heterologous gene into one of the six deletion sites of the MVA genome is clearly *not* the thrust of the Small *et al.* patent. This teaching can hardly be considered the thrust of Small *et al.* patent "since Small Jr. et al. do not specifically identify which insertion site is used in their recombinant viruses" (Office Action, page 6).

The Examiner states that the "insertion of sequences expressing antigens of HIV proteins into recombinant vaccinia viruses is specifically discussed in Example 4" and that "[t]his is a direct suggestion to create recombinant MVA viruses having heterologous sequences inserted into naturally occurring deletion sites" (Office Action, page 6).

Applicants respectfully disagree. It is not clear to Applicants how the Examiner finds such a "direct suggestion" in Example 4 of the Small *et al.* patent. In Example 4, Small *et al.* state that "Live recombinant ***vaccinia virus*** is prepared containing heterologous DNA sequences that encode various HIV antigens" (Small *et al.*, column 12, lines 3-5, emphasis added). Small *et al.* do not even refer to the MVA virus in Example 4. How does the Examiner know that the "live recombinant vaccinia virus" in Example 4 is MVA and not the

> deletion or insertion mutants of vaccinia virus, or a canary pox virus, (each of which is referred to herein generally as 'vaccinia' for ease of reference), that expresses a heterologous gene or genes (Small *et al.*, column 2, lines 30-33)?

The Examiner concludes that:

> it would have been obvious for one of ordinary skill in the art at the time the invention was made to have selected any one of the naturally occurring deletion sites of MVA for insertion of sequences encoding heterologous antigens. Small Jr. et al. disclose that MVA has six suitable sites for insertions, and indicates that any site can be utilized. Heterologous antigens are efficiently expressed from the insertion sites, and such antigens can provide protection from homologous challenge. Small Jr. et al. disclose the suitability of several antigens for such expression, including antigens of viruses, bacteria and parasites. One would have been motivated to use the MVA virus because it is an excellent vaccine candidate due to its extreme attenuation, the availability of insertion sites, the level of gene expression, and the safety for laboratory workers" (Office Action, page 6).

Applicants respectfully disagree. Small *et al.* disclose that MVA has six deletion sites. Small *et al.* do not indicate that any of the deletion sites can be used for insertions of sequences encoding heterologous antigens. In a section of the patent devoted to a discussion of the potential disadvantages of recombinant vaccinia viruses as human vaccines, Small *et al.* note that the risks associated with recombinant vaccinia viruses should be negligible with replication deficient vaccinia virus such as MVA. Small *et al.* then describe the general features of MVA, including the fact that "MVA suffered six major deletions of its genome, resulting in the loss of 30,000

base pairs . . . . so that it became host-restricted and unable to grow efficiently in mammalian cell lines"(Small *et al.*, column 6, lines 16-19). Small *et* al. do not mention the deletion sites again, not even in the exemplification in which MVA is used to prepare MVA HA-NP in Example 6. Small *et al.* do not teach that all six deletion sites are "suitable sites for insertions", and do not indicate that "any site can be utilized" (Office Action, page 6). The Examiner has pointed to no such teaching in the Small *et al.* patent.

As pointed out above, at the time of Applicants' invention it was known that heterologous genes could be inserted into non-essential regions of the vaccinia virus. A vaccinia virus genome comprises numerous non-essential gene which are used as insertion sites of foreign genes. It is likely that Small *et al.* inserted the heterologous gene into the C7L- locus, the tk-locus or one of the other 35 sites in the vaccinia virus genome into which foreign genes can be inserted, and which are also still present in the MVA geneome (see Table 1 of Smith, *Molecular Virology; A Practical Approach*, Eds. A.J. Davison and R.M. Elliot, IRL Press at Oxford University Press, a copy of which is being filed herewith as Exhibit A).

As pointed out in the previously filed Amendment, Small *et al.* administered MVA HA-NP to mice which demonstrated the "potential immunogenicity and efficacy of MVA HA-NP as an oral vaccine" (Small *et al.*, column 6, lines 63-64, Example 6). Small *et al.* do not specifically disclose how the MVA HA-NP was constructed. In the section on the general discussion of the MVA virus in the Small *et al.* patent (column 5, line 56 - column 6, line 38), Small *et al.* cite the Sutter *et al.* 1994 reference wherein plasmids in which the hemagglutinin (HA) and nucleoprotein (NP) genes are inserted into the deletion site III of the MVA virus are described (Sutter *et al.*, page 1032, column 2; Figure 1; Reference AR, PTO form 1449). ***However, Small et al. do not cite Sutter 1994 in Example 6 for the description of the MVA HA-NP (Small et al., column 12, line 34 - column 13, line 26)***. Small *et al.* do not teach or even suggest (other than the remote reference to Sutter *et al.* 1994 who inserted a gene into site III) that a foreign gene could be inserted into any naturally occurring deletion site within the MVA genome and expressed, and thus, do not provide a reasonable expectation of doing so.

Prior to Applicants' invention, it was known that the MVA genome had six major deletion sites and that all superfluous regions of the MVA genome (those not necessary for replication and generation of new virus) were deleted. At the time of Applicants' invention,

those of skill in the art found that the MVA deletion sites are located in variable, unstable regions of the genome. Meyer *et al.* 1991 (Reference AV on PTO form 1449) teach that some of the deletion sites are located in the terminal fragments of the MVA genome, and that both terminal regions undergo complex sequence rearrangement during cell propagation (Mayer *et al.*, page 1036, right column, lines 2-4). Meyer *et al.* also teach the presence of "long stretches of non-essential DNA" in deletion site regions of the MVA and assume that these sequence "encode a variety of proteins which interact with the host" (Meyer *et al.*, page 1036, left column). Such cDNA sequences are also called "transacting elements" and are necessary to reprogram host cells for viral replication and packaging of the viral genome into infectious particles. Accordingly, a person of skill in the art would expect that integrating heterologous sequences into regions of the genome which include a transacting element would preclude viral replication, and thus, generation of viral particles. Antoine *et al.* found that not only the terminal regions of the MVA genome are unstable, but that deletions also occur during cell propagation within the central genome region, indicating that this region is also unstable and undergoing sequence rearrangement (Antoine *et al., Virology, 244:*365-396 (1998), a copy of which is being filed herewith as Exhibit B, page 385).

Thus, at the time of Applicants' invention, those of skill in the art would expect that insertion of a heterologous sequence into MVA deletion sites, other than the MVA deletion site III of Sutter *et al.* 1994, would result in unstable integration (and thus, deletion of the heterologous sequence); rearrangement of the inserted sequence (and thus, non-expression of the heterologous sequence); and/or destruction of MVA transacting elements (and thus, prevention of the generation of viral particles expressing the heterologous sequence). Clearly, Applicants' data showing stable integration and expression of heterologous sequences in deletion sites other than site III of the MVA genome are surprising and unexpected results.

The obviousness rejection of record has been made with the advantage of impermissible hindsight, and thus, the rejection is legally improper. That is, in making the obviousness rejection, the Examiner has read the prior art with the benefit of Applicant's disclosure in which there is a clear teaching of a recombinant MVA virus containing and capable of expressing at least one foreign gene inserted at a site of a naturally occurring deletion within the MVA genome, wherein the site of the naturally occurring deletion is not site III, or wherein the site of

the naturally occurring deletion is selected from the group consisting of site I, II, IV, V and VI. As the court made clear in *In re Dow*, it is not legally correct to rely on Applicant's disclosure for the suggestion that the cited references should be combined and the expectation of success (*In re Dow Chemical*, 5 U.S.P.Q.2d 1529, 1531-1532 (Fed. Cir. 1988)).

Small *et al.* do not render obvious Applicants' claimed invention.

Rejection of Claims 6, 7, 31, 32, 39, 40, 48 and 49 under 35 U.S.C. §103(a)

Claims 6, 7, 31, 32, 39, 40, 48 and 49 under 35 U.S.C. §103(a) as being unpatentable over Small *et al.* in view of Altenberger *et al.* and further in view of Montagnier *et al.* The Examiner cites Smal *et al.* as above and adds that "Small Jr. et al. do not specifically identify which insertion site is used in their recombinant viruses, but it is noted that claims 32 and 33 do not specify where the heterologous gene is to be inserted" (Office Action, page 7). The Examiner further states that "[t]hose claims are not limited to naturally occurring deletion sites at all, let alone particular sites, and therefore are rendered completely obvious over the teachings set forth in the rejection" (Office Action, page 7). The Examiner states that Altenberger *et al.* disclose "recombinant MVA viruses" and "the location of deletion II"; suggest that "MVA recombinants can express malaria antigens from genes inserted into this location"; and note that "recombinant MVA viruses having insertions into the deletion II area could potentially be used as vaccines" (Office Action, page 7). The Examiner states that Altenberger *et al.* provide "direct suggestion and direct motivation to insert the foreign gene of interest (or any heterologous sequence) into the deletion II region of MVA, in order to obtain foreign gene expression" (Office Action, page 7). The Examiner states that Montagnier *et al.* disclose "the HIV nef protein, and nucleotides encoding nef, for use in producing recombinant nef polypeptides which can be used in HIV detection, and in immunogenic compositions", and express the nef protein from recombinant vaccinia viruses (Office Action, page 8). The Examiner states that "MVA is a specifically attenuated form of vaccinia virus, and thus, Montagnier *et al.* "provide direct motivation to express the nef protein in a vaccinia virus for use in vaccines" (Office Action, page 8). It is the Examiner's opinion that:

> It would have been obvious for one of ordinary skill in the art at the time the invention was made to have selected any one of the naturally occurring deletion

> sites of MVA, including site II, for insertion of sequences encoding heterologous antigens. Small Jr. et al. disclose that MVA has six suitable sites for insertions, and indicate that any site can be utilized. Both Altenberger et al. and Small Jr. et al. disclose that heterologous antigens are efficiently expressed from the insertion sites, and such antigens can provide protection from homologous challenge. Small Jr. et al. disclose suitability of several antigens for such expression including antigens of HIV for use in recombinant MVA viruses. One of skill in the art would have been motivated to select the nef gene of HIV in view of the disclosure of Montagnier et al., which indicates that immunogens comprising nef proteins are highly desirable for vaccine compositions against AIDS (Office Action, page 8).

Applicants respectfully disagree. As discussed above, Small *et al.* do not teach or even suggest that a foreign gene could be inserted into any naturally occurring deletion site, other than the remote reference to site III in the Sutter *et al.* 1994 reference, within the MVA genome and expressed.

Furthermore, Applicants respectfully disagree that Altenberger *et al.* provide "direct suggestion and direct motivation to insert the foreign gene of interest (or any heterologous sequence) into the deletion site II region of MVA" (Office Action, page 7). Altenberger *et al.* do not teach or even suggest that a foreign gene could be inserted into deletion site II of the MVA genome and expressed. Altenberger *et al.* analyzed the first and second deletion sites of the "strongly attenuated MVA virus as a necessary first step towards defining functions involved in attenuation" (Altenberger *et al.*, page 24) and found that "[a]lthough more than two thirds of the host range gene were eliminated from the MVA, the virus could still multiply in certain human cells" (Altenberger *et al.*, abstract). The Examiner refers to the teaching in Altenberger *et al.* that:

> [i]n the MVA virus this second deletion eliminated more than two thirds of the presumptive human host range. In spite of this, clinical vaccination trials [30] and our in vitro experiments strongly suggest that the MVA virus is able to replicate in certain human cells. Moreover, human 143 B TK-cells support the replication of MVA recombinant expressing malaria antigens (Altenberger *et al.*, page 25, first full paragraph).

However, this does not mean that Altenberger *et al.* inserted a malaria antigen in deletion site II of MVA. Here, Altenberger *et al.* is pointing out that despite the presence of deletion site II in the MVA virus, insertion of a malaria antigen in ***a common insertion site*** of MVA (the tk locus),

the virus could still multiply in certain human cells. See, for example, the reference by Altenberger *et al.* of "***MVA tk-recombinants***" in the following paragraph (Altenberger *et al.*, page 25, second full paragraph). If Altenberger *et al.* had made "MVA recombinants expressing malaria antigens" in which the antigen was inserted into deletion site II of MVA, they would have made more than a passing reference to it, since such MVA recombinants had not been made before and the expectation at the time was that such MVA recombinants would not successfully express foreign sequences.

As pointed out in the previously filed Amendment, Montagnier *et al.* teach "[p]olypeptides encoded by the nef gene of Human Immunodeficiency Virus (HIV)" (Montagnier *et al.*, abstract). Montagnier *et al.* further teach that the "nef protein can be obtained by expression of a sequence coding for the protein in vaccinia virus" (Montagnier *et al.*, column 13, lines 15-16). There is no discussion of the MVA virus in the Montagnier *et al.* patent.

The prior art combination of record has been made with the advantage of impermissible hindsight, and thus, the rejection is legally improper. That is, in making the obviousness rejection, the Examiner has read the prior art with the benefit of Applicant's disclosure in which there is a clear teaching of a recombinant MVA virus containing and capable of expressing at least one foreign gene inserted at a site of a naturally occurring deletion within the MVA genome, wherein the site of the naturally occurring deletion is not site III, or wherein the site of the naturally occurring deletion is selected from the group consisting of site I, II, IV, V and VI. As the court made clear in *In re Dow*, it is not legally correct to rely on Applicant's disclosure for the suggestion that the cited references should be combined and the expectation of success (*In re Dow Chemical*, 5 U.S.P.Q.2d 1529, 1531-1532 (Fed. Cir. 1988)). In the present case, the suggestion or motivation for combining the references and the expectation of success are not found in the prior art, but rather in Applicant's disclosure.

The combined teachings of Small *et al.* in view of Altenberger *et al.* and further in view of Montagnier *et al.* do not render obvious Applicants' claimed invention.

Rejection of Claims 6, 7, 33, 34, 39-43 and 48-52 under 35 U.S.C. §103(a)

Claims 6, 7, 33, 34, 39-43 and 48-52 are rejected under 35 U.S.C. §103(a) as being unpatentable over Small *et al.* in view of Altenberger *et al.* and further in view of Kwon. The

Examiner cites Small *et al.* and Altenberger *et al.* as above. The Examiner cites Kwon as disclosing "the cDNA sequence encoding human tyrosinase, and the expression of that protein from bacteriophage vectors" (Office Action, page 10). The Examiner states that Kwon "provides motivation to use the tyrosinase gene as an antigen as it is involved in melanomas, and could be a vaccine antigen" (Office Action, page 10). The Examiner states that:

> It would have been obvious for one of ordinary skill in the art at the time the invention was made to have selected any one of the naturally occurring deletion sites of MVA, including site II, for insertion of sequences encoding heterologous antigens. Small Jr. et al. disclose that MVA has six suitable sites for such insertions, and indicate that any site can be utilized. Both Altenberger et al. and Small Jr. et al. disclose that heterologous antigens are efficiently expressed from the insertion sites, and such antigens can provide protection from homologous challenge. Small Jr. et al. disclose the use of recombinant MVA viruses for cancer prevention when the proper cancer antigen is provided. Kwon provides that antigen, human tyrosinase, and indicates it could be used in a melonoma vaccine. One of skill in the art would have been further motivated to use the MVA virus because it is an excellent vaccine candidate due to its extreme attenuation, the availability of insertion sites, the level of gene expression, and the safety for laboratory workers (Office Action, page 10).

Applicants respectfully disagree. The claimed invention relates to a recombinant MVA virus containing and capable of expressing HIV nef or human tyrosinase inserted at a site of a naturally occurring deletion within the MVA genome, wherein the site of the naturally occurring deletion is not site III.

As discussed above, neither Small *et al.* nor Altenberger *et al.* teach or even suggest inserting a heterologous gene into a deletion site other than the remote discussion of Small *et al.* regarding the Sutter *et al.*, 1994 reference. Kwon does not provide what is lacking in the combined teachings of Small *et al.* and Altenberger *et al.* to render Applicants' claimed invention obvious. Kwon teaches "cDNA clones for human tyrosinase" (Kwon, column 1, line 20). There is no discussion of the MVA virus in the Kwon patent.

Applicants again submit that the prior art combination of record has been made with the advantage of impermissible hindsight, and thus, the rejection is legally improper. That is, in making the obviousness rejection, the Examiner has read the prior art with the benefit of Applicant's disclosure in which there is a clear teaching of a recombinant MVA virus containing

and capable of expressing at least one foreign gene inserted at a site of a naturally occurring deletion within the MVA genome, wherein the site of the naturally occurring deletion is not site III, or wherein the site of the naturally occurring deletion is selected from the group consisting of site I, II, IV, V and VI. As the court made clear in *In re Dow*, it is not legally correct to rely on Applicant's disclosure for the suggestion that the cited references should be combined and the expectation of success (*In re Dow Chemical*, 5 U.S.P.Q.2d 1529, 1531-1532 (Fed. Cir. 1988)). In the present case, the suggestion or motivation for combining the references and the expectation of success are not found in the prior art, but rather in Applicant's disclosure.

Accordingly, the pending claims are nonobvious over the cited references and their combination(s).

CONCLUSION

In view of the above amendments and remarks, it is believed that all claims are in condition for allowance, and it is respectfully requested that the application be passed to issue. If the Examiner feels that a telephone conference would expedite prosecution of this case, the Examiner is invited to call the undersigned at (978) 341-0036.

Respectfully submitted,

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Modified vaccinia virus Ankara undergoes limited replication in human cells and lacks several immunomodulatory proteins: implications for use as a human vaccine

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Modified virus Ankara (MVA) is a vaccinia virus (VV) strain that was attenuated by serial passage through chick embryo fibroblasts (CEFs) and contains six large genomic deletions compared with parental virus. MVA replicates well in CEFs, but poorly in most mammalian cells. Recombinant MVA is a promising human vaccine candidate due to its restricted host range, immunogenicity and avirulence in animal models, and excellent safety record as a smallpox vaccine. Here we present a further characterization of MVA and demonstrate that: (i) MVA can replicate, albeit poorly, in transformed human cell lines, but not in primary human fibroblasts although there is limited cell-to-cell spread; (ii) MVA is a potent inducer of type I interferon (IFN) from primary human cells, which may restrict virus spread *in vivo*; and (iii) unlike other VV strains, MVA does not express soluble receptors for IFN-γ, IFN-α/β, tumour necrosis factor and CC chemokines, but does express a soluble interleukin-1β receptor. This provides a plausible and testable explanation for the good immunogenicity of MVA despite its poor replication in mammals. The implications of these findings for the use of MVA as a safe and immunogenic human vaccine candidate are discussed.

Introduction

Recombinant poxviruses have been proposed as new vaccines. Concern about the safety of conventional smallpox vaccines has prompted the development of attenuated derivatives, avian poxvirus vectors, or the use of existing attenuated vaccinia virus (VV) strains such as modified virus Ankara (MVA). MVA was derived by > 500 passages in chick embryo fibroblasts (CEF) of material derived from a pox lesion on a horse in Ankara, Turkey (Hochstein-Mintzel *et al.*, 1972; Mayr *et al.*, 1975). It is highly attenuated yet induces protective immunity against veterinary orthopoxvirus infections (Mayr, 1976; Munz *et al.*, 1993). MVA was used in the final stages of the smallpox eradication campaign, being administered by intracutaneous, subcutaneous and intramuscular routes to more than 120000 humans in Southern Germany and Turkey. No significant side effects were recorded, despite the deliberate vaccination of high risk groups such as young, old or eczematous patients (Stickl *et al.*, 1974; Mayr *et al.*, 1978; Mahnel & Mayr, 1994). This safety in man is consistent with the avirulence of MVA in animal models, including neonatal and irradiated mice (Mayr *et al.*, 1978).

MVA has genomic deletions totalling 31 kb (Altenburger *et al.*, 1989; Meyer *et al.*, 1991) and produces small white pocks, rather than the large and ulcerated pocks produced by the parental virus cutaneous virus Ankara (Mayr *et al.*, 1975). Infection of most non-avian cells is abortive (Mayr *et al.*, 1978), partly due to deletions affecting host range genes K1L and C7L. However, some virus replication still occurs on human TK$^-$143B cells and African green monkey (CV-1) cells (Altenburger *et al.*, 1989) and restoration of the K1L open reading frame (ORF) only partially restores MVA host range (Meyer *et al.*, 1991; Sutter *et al.*, 1994*a*). Virus replication aborts late during infection of HeLa cells after immature virions are formed (Sutter & Moss, 1992), and this allows efficient expression of proteins by MVA even during abortive infection (Sutter & Moss, 1992; Wyatt *et al.*, 1995). Recombinant MVA expressing influenza virus nucleoprotein and haemagglutinin protected mice against a lethal challenge with influenza virus (Sutter *et al.*, 1994*b*). Likewise, recombinant MVA expressing simian immunodeficiency virus (SIV) antigens provided re-

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The sequence of the B8R gene from vaccinia virus strain MVA has been deposited in GenBank, accession number AF016273.

sistance to SIV-induced disease and afforded better protection than VV strain Wyeth (a current human vaccine) expressing the same SIV proteins (Hirsch *et al.*, 1995, 1996). Recombinant MVA also has potential as a tumour vaccine (Carroll *et al.*, 1997). In view of its safety and immunogenicity, recombinant MVA is considered a promising human vaccine candidate (Moss *et al.*, 1996).

Orthopoxviruses have evolved strategies for evasion of the host immune response that include the secretion of receptors for tumour necrosis factor (TNF) (Hu *et al.*, 1994; Smith *et al.*, 1996), interleukin (IL)-1β (Alcamí & Smith, 1992; Spriggs *et al.*, 1992), interferon (IFN)-γ (Alcamí & Smith, 1995; Mossman *et al.*, 1995), IFN-α/β (Colamonici *et al.*, 1995; Symons *et al.*, 1995) and CC chemokines (Graham *et al.*, 1997; C. A. Smith *et al.*, 1997; Alcamí *et al.*, 1998). These soluble receptors generally inhibit the host response, and in most cases their presence is associated with increased virulence. The IL-1β receptor (IL-1βR) is an exception: when virus was administered to mice by the intranasal route the IL-1βR diminished the signs of illness and weight loss, and prevented fever (Alcamí & Smith, 1992, 1996).

Here we report that MVA lacks functional receptors for TNF, IFN-γ, IFN-α/β and CC chemokines, but expresses the IL-1βR. In addition, we show that MVA undergoes limited replication in transformed human cells such as TK$^-$143B and HeLa cells, but not in primary human fibroblasts, although there is some evidence of cell-to-cell spread on immunostaining. In primary human cells MVA induces release of type I IFN. The significance of these results for the development of MVA as a human vaccine is discussed.

Methods

■ **Cells and viruses.** Human MRC-5, TK$^-$143B, HeLa and FS-2 cells (primary human foreskin fibroblasts) were provided by either the cell bank or P. Handford, Sir William Dunn School of Pathology, University of Oxford, UK. CEFs were prepared from whole embryo digests and grown in minimal essential medium (MEM) with 10% foetal bovine serum (FBS). Confluent monolayers of CEF were maintained at 31 °C for up to 4 weeks, with weekly changes of medium (MEM with 2% FBS). Fresh CEF monolayers were prepared the day before use by 1:3 split and overnight growth in MEM with 10% FBS.

The sources of VV strains Western Reserve (WR) and Lister, cowpox virus (CPV) strain Brighton Red and cocal virus have been described (Alcamí & Smith, 1995). MVA from human vaccine stock (II/85) and from passage 575 was kindly provided by A. Mayr, Veterinary Faculty, University of Munich, Munich, Germany. Unless otherwise stated, experiments relate to MVA II/85. MVA was routinely propagated and titrated in CEF grown in MEM. Recombinant baculovirus expressing the VV IL-1βR (AcB15R) or the VV IFN-α/β receptor (IFN-α/βR) (AcB18R) have been described (Alcamí & Smith, 1992).

■ **Immunostaining.** FS-2 cells were infected with MVA at 0·001 p.f.u. per cell and foci were stained 72 h post-infection (p.i.) with an anti-VV serum raised by live infection of a rabbit. Bound antibody was detected by peroxidase-conjugated polyclonal goat anti-rabbit (Sigma) then developed with diaminobenzidine (Sigma) in the presence of nickel sulphate and hydrogen peroxide.

■ **Cytokines.** Human recombinant ^{125}I-IL-1β (80 μCi/μg), ^{125}I-IL-1α (80 μCi/μg), ^{125}I-IFN-γ (90 μCi/μg), ^{125}I-RANTES (2200 Ci/mmol), ^{125}I-MIP-1α (2200 Ci/mmol) were obtained from Du Pont-England Nuclear. Human recombinant IFN-γ (1×10^7 units/mg) was from Genzyme and human natural IFN-α ($1{\cdot}5 \times 10^8$ units/mg) was obtained from Wellcome. Human recombinant IL-1β (2×10^8 units/mg), IL-1α, TNF-α (2×10^7 units/mg) were from R&D Systems, and IL-Iα (8×10^7 units/mg) was from Genzyme.

■ **Preparation of medium for binding and biological assays.** Unless otherwise stated, supernatants were harvested from CEFs 18 h p.i. with MVA at 5 p.f.u. per cell, or from TK$^-$143B cells or HeLa cells 48 h p.i. with CPV, VV strain WR or Lister at 10 p.f.u. per cell and were prepared as described (Alcamí & Smith, 1992). VV supernatants were inactivated with 4,5′,8-trimethylpsoralen and ultraviolet light for biological assays for IFN (Tsung *et al.*, 1996). Supernatants from insect cultures infected with recombinant baculovirus were prepared as described (Alcamí & Smith, 1992).

■ **Binding assays for cytokines, chemokines and interferons.** Soluble binding assays for IL-1βR and TNFR were performed by incubating 100 pM of ^{125}I-IL-1β or ^{125}I-TNF with culture supernatants and precipitation of the ligand–receptor complexes with polyethylene glycol and filtration through Whatman GF/C filters as described (Alcamí & Smith, 1992). Background radioactivity precipitated in the presence of medium was subtracted. Cross-linking experiments with 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) to ^{125}I-IFN-γ (2 nM), ^{125}I-RANTES (0·4 nM) or ^{125}I-MIP-1α (0·4 nM) were performed in 20 μl as described (Alcamí & Smith, 1995; Alcamí *et al.*, 1998). Samples were analysed by electrophoresis in 14% polyacrylamide gels.

■ **Activity assay for IFN.** The biological activity of human IFN was assayed by its ability to inhibit plaque formation by cocal virus (a rhabdovirus related to vesicular stomatitis virus) in cultures of HeLa cells as described (Alcamí & Smith, 1995). Cell monolayers were pre-treated for 16–24 h with IFN and virus-free supernatants, infected with approximately 100 p.f.u. of cocal virus, and plaques counted 48 h p.i.

■ **Cloning and sequencing of B8R gene and flanking regions.** The B8R gene and flanking regions were amplified from MVA DNA by PCR using *Pyrococcus furiosus* polymerase and the primers 5′ CTAGAATTCAACGCAGAGGTCACACG 3′ and 5′ TCAAAGCTTCACTTGCAGTTGGG 3′. The products of four separate PCR reactions were cloned into pCR-Script (Stratagene) and sequenced on both strands.

Results

MVA has a restricted replication in mammalian cells, yet is a potent immunogen inducing immune responses equal to or greater than those induced by fully replication competent VV strains such as WR or Wyeth. Since many orthopoxviruses secrete proteins from infected cells that interfere with the host response to infection and which might diminish virus immunogenicity, we wondered if the enhanced immunogenicity of MVA reflected the loss of these immunomodulatory proteins. To assess this we performed binding assays or biological assays for receptors for IL-1β, TNF, IFN-α/β, IFN-γ and CC chemokines in the supernatant of cells infected with MVA.

Cytokine receptor profile of MVA

To determine if MVA expressed an IL-1βR, supernatants from mock- or virus-infected CEFs were tested in a soluble

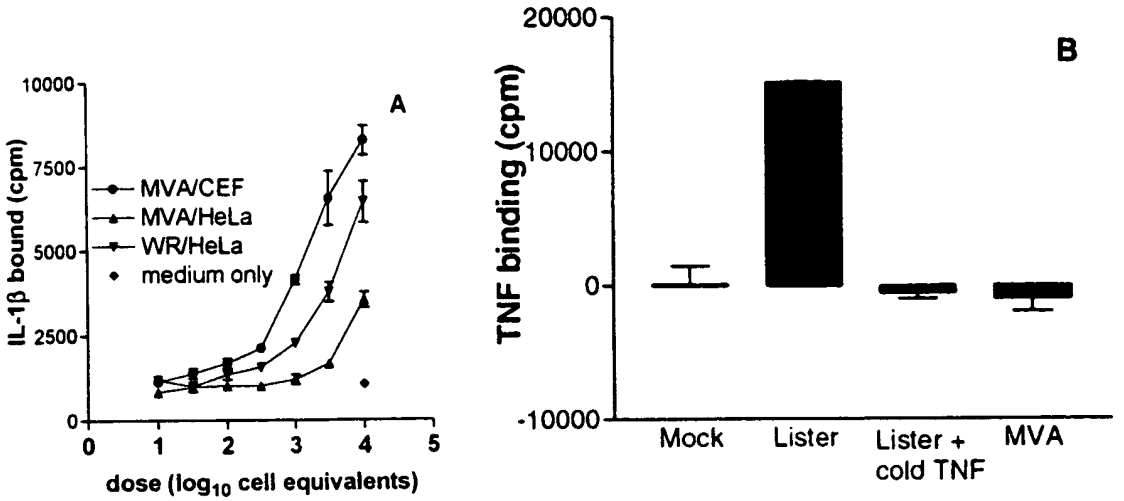

Fig. 1. MVA expresses a soluble IL-1βR but not a TNFR. (*a*) IL-1β binding assay. Supernatants of CEFs or HeLa cells infected with MVA at 5 p.f.u. per cell (harvested at 18 h p.i.), or from HeLa cells infected with VV WR were then incubated with ^{125}I-IL-1β and complexes were measured (Alcamí & Smith, 1992). (*b*) TNF binding assay. The supernatants from 1×10^5 TK$^-$143B cells infected with VV strain Lister, or mock-infected, or the same number of CEFs infected with MVA, were incubated with human ^{125}I-TNFα and bound TNF was determined as for IL-1β in (*a*). The specificity of the TNF-α binding by the supernatants of TK$^-$143B cells infected with VV strain Lister was confirmed by cold competition with a 100-fold excess of cold TNF-α. Data shown ± SEM ($n = 2$).

binding assay with human ^{125}I-IL-1β. Fig. 1(*a*) shows that CEFs infected with MVA secrete an IL-1β binding activity that could be detected in the supernatant from only 200 cells. An IL-1βR was produced by HeLa cells infected with VV strain WR, but also by the non-replicating MVA. As the B15R gene encoding the IL-1βR of VV strain WR is expressed late during infection (Alcamí & Smith, 1992), this result was consistent with the report that MVA late virus proteins are expressed in HeLa cells (Sutter & Moss, 1992). Notably, the level of IL-1βR made in MVA-infected HeLa cells was lower than that in WR-infected HeLa cells or in MVA-infected CEFs. Competition experiments with excess cold human IL-1β or IL-1α showed the MVA IL-1R was specific for IL-1β (data not shown), as for other VV strains (Alcamí & Smith, 1992, 1996).

The expression of TNFR was examined next. Although VV strains Copenhagen and WR contain ORFs (C21L/B28R and A53R) with amino acid similarity to TNFR (Smith *et al.*, 1990; Howard *et al.*, 1991; Upton *et al.*, 1991), these ORFs are interrupted and non-functional. In fact, TNFRs are not expressed by the majority of VV strains and only by a few strains including Lister (A. Alcamí and others, unpublished data). Fig. 1(*a*) shows that MVA-infected CEFs produced no TNFR. In contrast, TK$^-$143B cells infected with VV strain Lister secreted a TNF-α binding activity that was competed with excess unlabelled TNF-α. TNF binding activity was detected in only 1 μl of supernatant (data not shown), whereas 100 μl from MVA-infected cells had no binding activity.

Recently, some leporipoxviruses and orthopoxviruses were shown to express a soluble chemokine binding protein (CKBP) (Graham *et al.*, 1997; C. A. Smith *et al.*, 1997; Alcamí *et al.*, 1998) unrelated to cellular chemokine receptors which are 7 transmembrane G protein coupled proteins. Graham *et al.* (1997) reported that the protein bound CC and CXC chemokines, while using biological assays we showed a specificity for only CC chemokines (Alcamí *et al.*, 1998). To determine if MVA expressed a CC CKBP, human ^{125}I-labelled RANTES or MIP-1α was incubated with the supernatants of MVA-infected CEFs in the presence of a chemical cross-linker, followed by electrophoresis and autoradiography (Fig. 2). Cells infected with VV strain Lister or CPV expressed a CKBP which bound both these human CC chemokines and was detected with only 1 μl of supernatant, and as previously noted the size of the CPV protein was smaller than that from VV Lister (Alcamí *et al.*, 1998). In contrast, no CC chemokine binding activity was detected in 20 μl of supernatant of mock-infected cells or cells infected with VV WR, MVA 575 or MVA II/85.

MVA lacks IFN-γ and IFN-α/β receptors

Most VV strains express a type I and type II IFNR (Alcamí & Smith, 1995; Symons *et al.*, 1995). The presence of these proteins in MVA-infected cell supernatants was therefore examined by cross-linking with human ^{125}I-IFN-γ (Fig. 2) and bioassays for type I and type II IFNs (Fig. 3). The supernatants from cells infected with CPV and VV WR and Lister each expressed a protein which bound ^{125}I-IFN-γ (Fig. 2) and, as previously noted (Alcamí & Smith, 1995), the CPV protein was slightly smaller than that expressed by either VV strain. In contrast, neither MVA isolate expressed an IFN-γR. This result was confirmed with a biological assay. Pre-treatment of HeLa cells with human IFN-γ or IFN-α inhibits plaque formation by cocal virus. However, the antiviral activity of these IFNs was reversed by pre-incubating IFN with the supernatant from CPV-infected cells (Fig. 3, CPV). CPV is known to express an

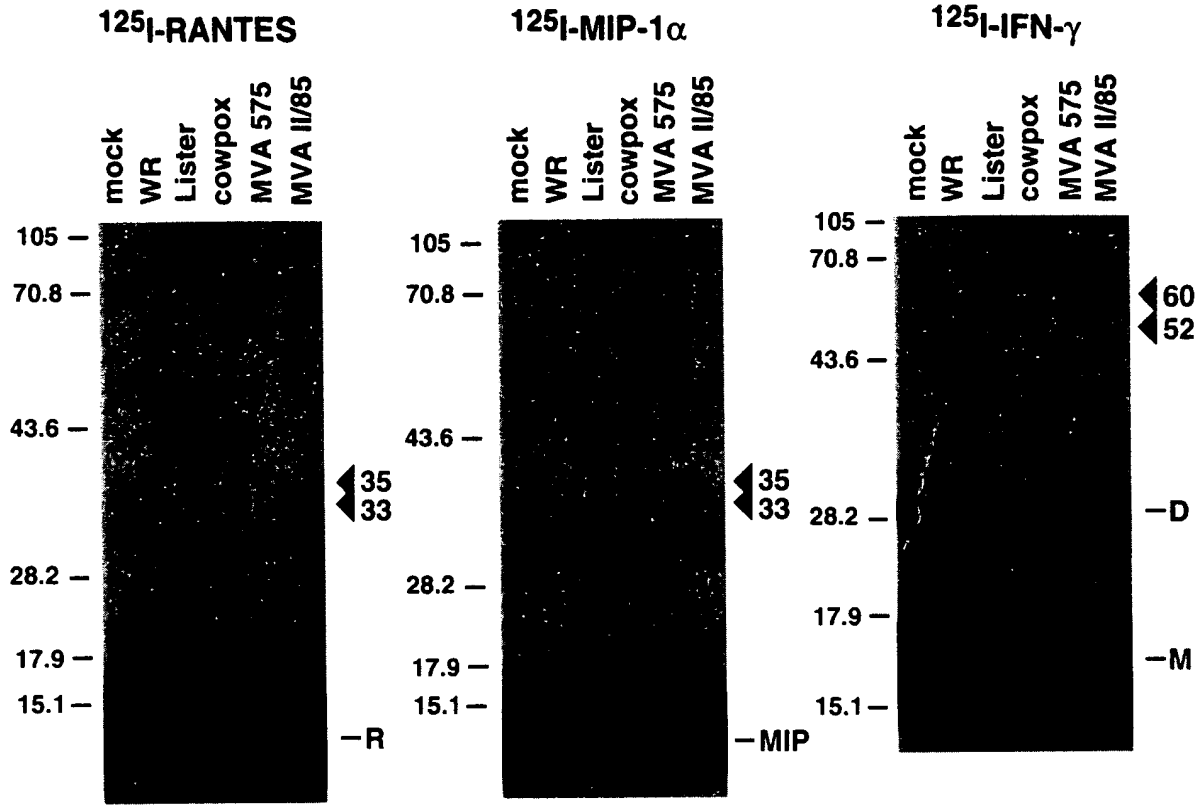

Fig. 2. MVA does not express an IFN-γR or a CC CKBP. Supernatants from 4×10^4 TK$^-$143B cells uninfected (mock) or infected with CPV, VV Lister or VV WR or from the same number of CEFs infected with MVA 575 or MVA II/85 were incubated with ^{125}I-IFN-γ, ^{125}I-RANTES or ^{125}I-MIP-1α. Complexes were cross-linked with EDC, resolved from monomeric or dimeric ligands by PAGE and detected by autoradiography. The positions of IFN-γ monomers (M), IFN-γ dimers (D), RANTES (R), MIP-1α (MIP) and ligand receptor complexes (arrowheads) are indicated. Molecular masses in kDa are shown.

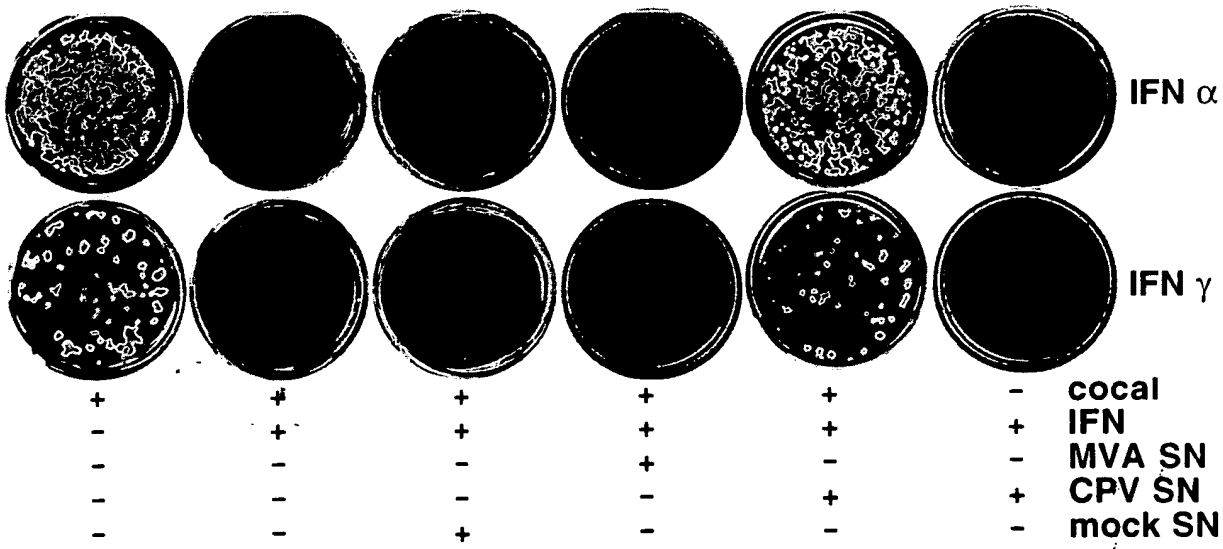

Fig. 3. Bioassay for type I and type II IFNRs. HeLa cell monolayers in 24-well plates were infected with approximately 100 p.f.u. of cocal virus in the presence of supernatant from 10^5 CEFs that had been infected with MVA, or from TK$^-$143B cells that had been either mock-infected or infected with CPV. Either 50 U/ml of human IFN-α or 20 U/ml of human IFN-γ or no IFN were included in the overall medium containing 1·5% (w/v) carboxymethylcellulose as indicated. Cells were stained with 0·1% crystal violet in 15% ethanol 48 h p.i. The presence of IFNR activity is demonstrated by reversal of IFN-induced suppression of cocal virus plaque formation.

(a)

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MVA MRYIIILAVLFINSIHAKITSYKPESVNFDSKIEWTGDGLYNISLKNYGIKTWQTMYTIKTWQTMYTNVPEGTYDISAFPKNDFVSFWVKPE
WR  MRYIIILAVLFINSIHAKITSYKPESVNFDSKIEWTGDGLYNISLKNYGIKTWQTMYTIKTWQTMYTNVPEGTYDISAFPKNDFVSFWVKPE

MVA QGDYKVEEYCTG--------PPTVTLTEYDDH-------PYATRGSKKIPIYKRGDMCDIYLLYTANFTFGDSKEPVPYDIDDYDCTSTGCSIDF
WR  QGDYKVEEYCTGLCVEVKIGPPTVTLTEYDDHINLYIEHPYATRGSKKIPIYKRGDMCDIYLLYTANFTFGDSKEPVPYDIDDYDCTSTGCSIDF

MVA VTTEKVCVTAQGATEGFLEKITPWSSKVCLTPKKSVYTCAIRSKEDVPNFKDKMARVIKRKFN
WR  VTTEKVCVTAQGATEGFLEKITPWSSKVCLTPKKSVYTCAIRSKEDVPNFKDKMARVIKRKFNKQSQSYLTKFLGSTSNDVTTFLSMLNLTKYSM
    A                                  N
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(b)

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MVA      GGAC------------------------CACC
WR 28837 GGACTATGCCTCGAAGTAAAAATTGGACCACC 28868
MVA      GACCAT--------------------CCGTAT
WR 28893 GACCATATCAATTTGTACATCGACGCATCCGTAT 28926
MVA      TTTAA-----------------------CTAAA
WR 29272 TTTAATAAACAGTCTCAATCTTATTTAACTAAA 29304
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Fig. 4. B8R sequence comparisons. (*a*) Alignment of predicted protein sequences for B8R of MVA and VV WR, showing effects of three deletions within the ORF. Note that the third deletion introduces a frameshift resulting in termination immediately downstream. The two amino acid changes between the B8R proteins in the WR and Copenhagen strains of VV are indicated underneath the WR sequence. (*b*) Nucleotide sequence alignments for MVA and WR showing the three deletions within the B8R ORF. Short direct oligonucleotide repeats flanking the deletions are underlined. Numbers refer to the nucleotide positions within a 42 kbp region of VV strain WR (Smith *et al.*, 1991).

IFN-γR and IFN-α/βR that neutralize these human IFNs (Alcamí & Smith, 1995; Symons *et al.*, 1995). No such inhibitory activity was expressed from MVA-infected CEFs (Fig. 3, MVA) or mock-infected cells.

Although MVA did not express an IFN-γR, PCR analysis using primers flanking the VV WR B8R gene amplified an MVA DNA fragment of similar size to that of VV WR (data not shown). The MVA IFN-γR locus was therefore sequenced and compared with the VV WR B8R gene (Smith *et al.*, 1991). The sequences were identical except for three silent nucleotide substitutions and three short deletions in MVA. The first two deletions eliminated 24 or 21 nucleotides and therefore retained the ORF while removing 8 or 7 amino acids, respectively (Fig. 4*a*). The third deletion of 23 nucleotides caused a translational frameshift and introduced a termination codon immediately downstream. Consequently, the B8R protein of MVA is truncated by 32 amino acid residues. It is uncertain which of these mutations is likely to cause the loss of IFN-γ binding activity, but it is noteworthy that (i) the first deletion eliminates a conserved cysteine residue that might be important for the structural integrity of the B8R protein and (ii) the C terminus of the protein is outside the type III fibronectin repeats that are typical of the class II cytokine receptor family to which these poxvirus IFN-γRs belong. The MVA B8R gene is likely to be transcribed at levels comparable to VV WR since the promoter region of each virus is identical in sequence.

An interesting feature of the deletions is that each is flanked by short direct repeats of 4 to 6 nucleotides (although repeat 2 has matches at only five out of six positions) (Fig. 4*b*). Similar short repeats occur at many of the positions where the sequence of VV strain WR has short differences in length with VV Copenhagen (Smith *et al.*, 1991) or variola virus strain Harvey (Aguado *et al.*, 1992). These direct repeats are likely to be the cause of heterogeneity in orthopoxvirus genomes.

MVA can replicate in transformed but not primary human cells

MVA replicates poorly in most mammalian cells but well in avian cells such as CEFs. In human cells, MVA has been reported to replicate in TK$^-$143B cells (Altenburger *et al.*, 1989) but not in HeLa cells (Meyer *et al.*, 1991; Sutter & Moss, 1992). If recombinant MVAs are to be used for immunoprophylaxis or immunotherapy in humans, it is important to establish whether or not the virus can replicate in human cells. Therefore, we examined MVA replication in four human cell types and compared these to CEFs and mouse BALB3t3 cells (Fig. 5*a*). The human cells selected were two transformed cell lines (HeLa and TK$^-$143B) and two non-transformed or primary cell cultures (MRC-5 and FS-2). Infections were performed at 0·05, 0·5 and 5 p.f.u. per cell and the data are expressed as the increase or decrease in titre at 48 h p.i. relative to the input virus. At each m.o.i. there was a net increase in virus titre in HeLa and TK$^-$143B cells but a decrease in MRC-5 and FS-2 cells. The increase in titre in TK$^-$143B cells was expected, but our data showed a more modest increase than the 30-fold increase reported previously (Altenburger *et al.*, 1989). The small increase in HeLa cells was contrary to previous reports (Meyer *et al.*, 1991; Sutter & Moss, 1992) but was reproducibly observed. In non-transformed human cells there was a consistent 5- to 10-fold drop in titre. Likewise, infection of mouse BALB3t3 cells consistently yielded less virus than input.

MVA can induce focus formation on FS-2 cells

Although MVA did not increase in titre in FS-2 cells, the fact that the virus titre at 48 h p.i. was only 5-fold lower than the original virus input suggested that some limited virus

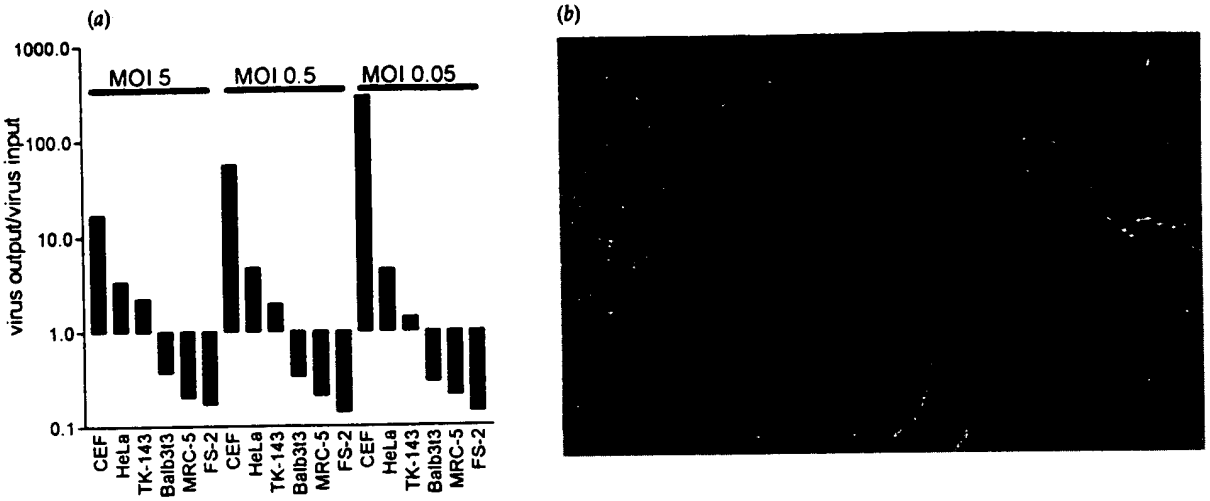

Fig. 5. MVA replication on chick, human and murine cells. (*a*) The indicated cells were infected with MVA at 5, 0·5 or 0·05 p.f.u. per cell for 2 h, unbound virus was removed by washing and cells were incubated in fresh medium for 48 h. Infected cells were scraped into the medium, frozen and thawed three times, sonicated and diluted for titration on fresh CEFs. Virus present before or after infection was titrated by immunostaining foci of infected cells with anti-VV antibody 72 h p.i. (*b*) Focus of infection showing evidence of cell-to-cell spread. FS-2 cells were infected with MVA at 0·001 p.f.u. per cell for 72 h and then stained with a rabbit antibody raised against a live VV infection (diluted 1:125) followed by a goat anti-rabbit–peroxidase conjugate and diaminobenzidine.

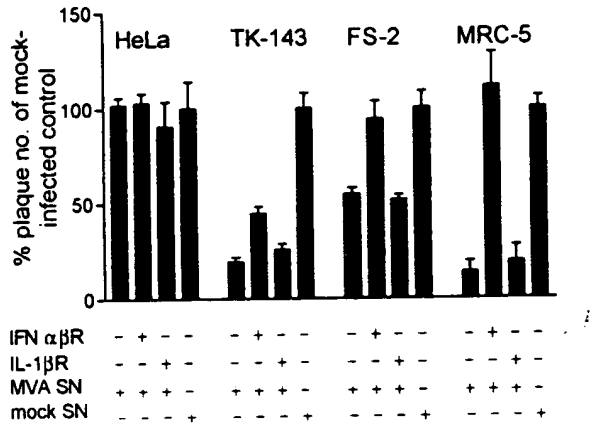

Fig. 6. Induction of type I IFN by human cells infected with MVA. Monolayers of the indicated cells were either mock-infected or infected with MVA for 24 h in 2 ml of MEM with 2% FBS. The supernatants were harvested and mixed with 100 p.f.u. of cocal virus in the presence of 50 µl of either the VV WR IFN-α/βR or IL-1βR, each produced from recombinant baculovirus infected cells (Alcamí & Smith, 1992). Mixtures were used to infect HeLa cells which were overlaid with medium containing 1·5% (w/v) carboxymethylcellulose and stained with crystal violet 48 h p.i. Data are expressed as the percentage of plaques formed compared to those formed in the presence of medium from mock-infected cells. The means ± SD ($n = 4$) are indicated.

replication might have taken place. Without any virus replication, the titre of virus present at 48 h p.i. would be expected to be orders of magnitude lower due to the uncoating and loss of infectivity of the great majority of the input virus. We therefore assessed whether MVA could spread from the initial infected cell by infecting FS-2 cells at 0·001 p.f.u. per cell and staining with a polyclonal anti-VV serum 3 days later (Fig. 5*b*). Foci (more than two adjacent staining cells) were detected where VV antigen had spread to surrounding cells, although 98% of infected cells were infected in isolation. This was consistent with virus replication and spread from a limited number of infected FS-2 cells.

MVA is a potent inducer of type I IFN

To investigate why MVA was replicating in transformed but not primary human cells, the ability of these cells to produce type I IFN after MVA infection was assessed (Fig. 6). Type I IFN released from these cells would remain active because MVA does not express a type I IFNR (Fig. 3). The presence of IFN in these supernatants was measured by inhibition of plaque formation by cocal virus on HeLa cells. HeLa cells infected by MVA produced no activity that inhibited cocal virus replication in fresh HeLa cells. In contrast, supernatant from MVA-infected TK$^-$143B, FS-2 and MRC-5 cells all contained an inhibitor. This was partly (TK$^-$143B cells) or completely (FS-2 and MRC-5 cells) mediated by type I IFN, because addition of the VV WR soluble IFN-α/βR, but not soluble IL-1βR, expressed from recombinant baculovirus (Alcamí & Smith, 1992; Symons *et al.*, 1995) reversed this inhibition (Fig. 6). The most dramatic result was seen with MRC-5 cells where MVA infection induced sufficient type I IFN to inhibit cocal virus plaques by approximately 90% and this inhibition was completely reversed by addition of the VV IFN-α/βR. The inhibition was only approximately 50% using supernatants from MVA-infected FS-2 cells, but as for MRC-5 cells, this was completely reversed by the IFN-α/βR. The situation with TK$^-$143B cells was more complex because although MVA-infected TK$^-$143B cell supernatants inhibited

cocal virus formation by approximately 80%, this was only partially reversed by the IFN-α/βR, suggesting another inhibitor was present.

The induction of type I IFN by MVA infection of primary human fibroblasts and the lack of an MVA encoded IFN-α/βR suggested that production of IFN might explain why MVA replication in these primary human fibroblasts was abortive. However, incubation of either MRC-5 or FS-2 cells with VV IFN-α/βR from recombinant baculovirus throughout infection with MVA did not enable an increase in virus titre (data not shown). Thus other factors restrict MVA replication in these cells.

Discussion

MVA is a severely attenuated VV strain that has safely prevented orthopoxvirus infections in man and animals. MVA is unable to replicate in most mammalian cell lines, but despite this is more immunogenic than replication competent vaccinia virus Wyeth in animal models. Consequently, MVA is a very promising candidate antigen delivery vehicle for immunoprophylaxis and immunotherapy that is being developed as an alternative to conventional VV strains or recombinant avipoxviruses. It is important to understand better why MVA is so immunogenic despite its attenuation and to examine further the replicative potential of MVA in human cells. This is especially important given the potential to vaccinate people who may be immunosuppressed due to HIV infection. In this paper we have addressed both these issues and show that MVA (i) lacks several of the immunomodulatory proteins expressed by many other orthopoxviruses, (ii) can replicate poorly in some human cells and (iii) can induce production of type I IFN from primary human fibroblasts.

Following the development of recombinant poxviruses as expression vectors and potential live vaccines in 1982, several laboratories characterized VV virulence genes in order to attenuate the virus and make it a safer vaccine. This work identified many VV proteins that interfere with the host response to infection (for review see G. L. Smith *et al.*, 1997). A group of these proteins function as soluble inhibitors of cytokines, chemokines and IFNs. These prevent their ligands reaching the cellular receptors and triggering the inflammatory and immune response to infection. By studying the roles of these immunomodulatory proteins in VV and other systems, we consider that the presence of the IL-1βR and the absence of receptors for IFN-α/β, IFN-γ, TNF and CC chemokines might represent a suitable profile for a safe and immunogenic orthopoxvirus vaccine. The expression of these proteins in MVA was therefore characterized and revealed that MVA has exactly this profile. Moreover, out of the 16 strains of VV that we examined (buffalopox, Copenhagen, Dairen, Evans, IHD-J, IHD-W, King Institute, Lister, MVA, Patwadangar, rabbitpox, Tashkent, Tian Tan, USSR, WR, Wyeth) MVA is the only strain with such a profile (Alcamí & Smith, 1992, 1995, 1996; Symons *et al.*, 1995; Alcamí *et al.*, 1998; A. Alcamí and others, unpublished data).

The loss of the IFN-γR is rare in VV strains (Alcamí & Smith, 1995), implying that this is an important protein for VV. IFN-γ is important in promoting the Th-1 type response to infection that includes the induction of cytolytic T cells. A virus lacking an IFN-γR is thus likely to be better recognized by the immune system and therefore be less virulent, while at the same time be able to induce a stronger immune response and therefore be more immunogenic. The virulence and immunogenicity of VV strains lacking the IFN-γR have not been reported, but a myxoma virus mutant lacking the IFN-γR is attenuated (Mossman *et al.*, 1996), although this attenuation could also be due to binding of chemokines by this protein (Lalani *et al.*, 1997). Although the lack of an IFN-γR is probably an important factor contributing to the attenuated and immunogenic phenotype of MVA, other immunomodulatory molecules may play a role. This is implied by the demonstration that MVA is severely attenuated in mice (Mayr *et al.*, 1978), a host in which the VV IFN-γR is ineffective (Alcamí & Smith, 1995).

The loss of the IFN-α/βR may also be quite important due to the ability of MVA to induce a type I IFN response, the established role of the IFN-α/βR as a factor promoting VV virulence (Colamonici *et al.*, 1995; Symons *et al.*, 1995), the ability of type I IFN to have direct antiviral activity and to enhance immunological memory (Tough *et al.*, 1996), and the fact that the only other VV strains which lack the IFN-α/βR (Lister) or express a receptor with a greatly reduced affinity for human IFN-α2 (Wyeth) were among the safer human smallpox vaccines (Fenner *et al.*, 1988). Lack of a TNFR, which has only been shown to decrease myxoma virus virulence (Upton *et al.*, 1991), is also likely to enhance MVA safety and immunogenicity due to the direct antiviral activity and pro-inflammatory activity of this cytokine. However, it may not explain the enhanced immunogenicity of MVA compared to many other VV strains, since the majority of these strains do not express TNFRs (A. Alcamí and others, unpublished data). The loss of a soluble inhibitor of a broad range of CC chemokines, which induce migration of leukocytes to sites of infection, is also likely to restrict virus replication in infected tissues and possibly enhance its immunogenicity.

The expression of an IL-1βR by MVA is interesting and consistent with the observation that (i) expression of an IL-1βR by other VV strains prevents fever and reduces virulence in mice (Alcamí & Smith, 1992, 1996), and (ii) IL-1βRs were produced by the safer smallpox vaccines (Alcamí & Smith, 1992). However, these observations were made in the context of severe experimental infection or human vaccination with fully replication competent VV. It is possible that due to the very limited replication of MVA, the IL-1βR might have little effect upon virulence and possibly suppress the immune response. IL-1β has been reported to increase the primary immune response (Plebanski *et al.*, 1992) and thus deletion of

this gene might enhance the immunogenicity of MVA. Potent immunogenicity of viruses, such as MVA, with severely restricted replication in vaccinees, and thus low level of antigen production, may be necessary for efficacy.

It is quite possible that there are mutations in the MVA genome other than those reported here which influence immunogenicity and host range. The precise roles of the virus immunomodulatory proteins described here will require the re-introduction or deletion of these genes from the MVA genome and direct comparisons of immunogenicity. However, given our knowledge of the function of the ligands bound by these molecules, the virus proteins are excellent candidates to influence the host response to infection.

The limited replication of MVA in some human cells is relevant to its potential use as a human vaccine. The virus titre increased in transformed human cells (TK$^-$143B and HeLa) but decreased after infection of primary human fibroblasts (MRC-5 and FS-2). Even in primary fibroblasts it is possible that infectious particles are produced in some cells because after infection of FS-2 cells at low multiplicity, foci of infection were visible after immunostaining with anti-VV antibody. The fact that only a small proportion (approximately 2%) of infected cells yielded foci suggests that this was a stochastic event caused by virion replication and spread rather than translocation of virus antigen. The inability to replicate well in primary human cells might be attributable to several factors including loss of the human host range gene and production of type I IFN, although replication in human cells was not restored after addition of a soluble inhibitor of human type I IFN. In addition, in HeLa cells MVA shows similar sensitivity to type I IFN as VV strain WR lacking the type I IFNR (data not shown). This suggests that intracellular modulators of IFN such as the E3L and K3L gene products might be expressed by MVA, but further work is required to confirm this. The ability of MVA to induce type I IFN, while not expressing an IFN-α/βR, would be a desirable feature *in vivo* that would restrict MVA replication and probably enhance vaccine safety and immunogenicity. Importantly, IFN induction would still occur in vaccinees that had an impaired immune system.

In summary, this study provides a further characterization of MVA and shows that this virus can replicate poorly in transformed human cell lines, but not in primary human fibroblasts where it is a potent inducer of type I IFN. Unlike other VV strains, MVA does not express soluble proteins that bind IFN-γ, IFN-α/β, TNF and CC chemokines, but does express a soluble IL-1β receptor. This provides a plausible explanation for the good immunogenicity of MVA despite its poor replication in mammals. Lastly, the B8R locus is identified as another locus into which foreign antigens or adjuvant molecules may be inserted for expression by recombinant MVAs.

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